Attorney Docket No. 0088562-033US0 PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Applicant | : | NYKJAER, Anders |
| Appl. No. | : | 10/539,443 |
| Filed | : | June 20, 2005 |
| For | : | MODULATION OF ACTIVITY OF NEUROTROPHINS |
| Examiner | : | MACFARLANE, Stacey Nee |
| Group Art Unit | : | 1649 |
| Confirmation No. | : | 6823 |

**DECLARATION UNDER 37 C.F.R §1.132**

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, Eero Castrén, do declare and state as follows:

1. I am the Sigrid Jusélius Professor in neuroscience and a Research Director at the University of Helsinki. I hold an M.D. as well as a Ph.D. in the field of neuroscience and neuropharmacology.

2. My research relates to the physiological and pathophysiological roles of neuronal plasticity and neutrotrophins in the adult brain. I am familiar with experimental laboratory and clinical research, particularly those related to elucidating the role and mechanism of compounds in affecting neurotophin signaling in normal brain development as well as in neurodevelopmental and neuropsychiatric disorders of the brain.

3. Mature neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NR4/5) mediate cell survival through formation of a ternary complex with $p75^{NTR}$ and the receptors Trk A/B/C. *See*, for example, Yano *et al.* (2000. *Pharmaceutica Acta Helvetiae* 74:253-260), Airaksinen *et al.* (2002. *Nature Reviews – Neuroscience* 3:383-394), Dechant *et al.* (2001. *Cell Tissue Res.* 305:229-238), Chao *et al.* (2003. *Nature Reviews – Neuroscience* 4:299-309) and Bibel *et al.* (1999. *EMBO J* 18(3):616-622).

4. Treatment of experimental models of various diseases and preliminary trials to clinically treat these diseases by administration of neurotrophins is known in the art. For example, use of NGF has been demonstrated in preliminary clinical trials for treatment of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.

5. The clinical importance of pro-neurotrophins (or neurotrophin precursors) in promoting apoptosis of neurons, which results in diseases associated with neuron degeneration or damage, is also known in the art (Lee *et al.* 2001. *Science* 294:1945-1948).

6. I have reviewed and evaluated the data presented by the Applicant, which are in Appendices A and B.

7. It is my understanding that the data in Appendix A relate to an *in vivo* experiment, in which male rats were subjected to spinal cord injury in the absence or presence of the pro-domain of pro-NGF (Appendix A).

8. I understand that the pro-domain of NGF used binds sortilin, which is a Vps10p-domain receptor. I also understand that the pro-domain acts as a sortilin antagonist and prevents pro-neurotrophins from binding to the receptor sortilin.

9. The data illustrated in Appendix A demonstrate that in the absence of the pro-domain of proNGF, when a greater amount of unbound sortilin was available, 49% of the injured spinal cord neurons survived.

10. The data in Appendix A also demonstrate that in the presence of the pro-domain sortilin antagonist, which resulted in binding of available sortilin, 67% of the injured spinal cord neurons survived.

11. In my view, the results of these *in vivo* mouse experiments indicate that blockade of the sortilin receptor by the pro-domain antagonist has a survival effect on neuronal injury.

12. Based on the *in vivo* data presented in Appendix A, I conclude that inhibition of binding between pro-neurotrophins and sortilin can be effective in methods for treating injury or dysfunction of the nervous system.

13. Recent publications also confirm that Vps10p domain receptors, such as sortilin, play a role in controlling neuronal survival or cell apoptosis.

14. Pro-NGF-$p75^{NTR}$-sortilin signaling has been identified to influence selective cell death of neurons, that are destroyed in Parkinson's Disease (Chen *et al.* 2008. *CNS Neurol Disord Drug Targets* 7(6):512-523).

15. It has also been demonstrated that the use of neutralizing antibodies that bind the pro-domain of pro-BDNF, or that bind to sortilin, reduces the death of injured sensory neurons *in vitro*. The use of neutralizing antibodies *in vivo* has also been observed to increase the number of sensory neurons in the central nervous system (Fan *et al.* 2008. *Eur J Neurosci* 27(9):2380-2390).

16. The presence of sortilin has been observed in basal forebrain neurons, and it has been found that the use of antibodies against sortilin is effective in preventing pro-neurotrophin-induced cell death of basal forebrain neurons, which are destroyed in Alzheimer's Disease (Volosin *et al.* 2006. *J Neurosci* 26(29): 7756-7766).

17. Thus, taken together, the results illustrated in Appendix A and the art (as exemplified by the publications cited in paragraphs 14-18) clearly support a role for Vps10p-domain receptor signaling in the pathology of injury or dysfunction of the central and peripheral nervous system.

18. In addition, it is my understanding that in an *in vitro* experiment, a rabbit anti-sortilin antibody (Nykjaer *et al.* 2004. *Nature* 427:843-848) was developed by the inventor and tested for the ability to inhibit binding between a pro-neurotrophin (pro-NGF) and sortilin (Appendix B).

19. I understand that in the experiment, recombinant human sortilin was immobilized on a BIAcore sensorchip, and buffer with or without 10μg/ml rabbit anti-sortilin antibody (IgG) was applied to separate flow cells on the chip.

20. It is my understanding that after application of the buffer (with or without anti-sortilin antibody), pro-NGF was applied to the separate flow cells, and association of pro-NGF binding was measured for 500 seconds.

21. In my view, the data illustrated in Appendix B demonstrate that in the flow cell that was pre-incubated with the rabbit anti-sortilin antibody, no binding was observed between pro-NGF and the immobilized sortilin.

22. In contrast, in the flow cell that was pre-incubated with buffer alone, a strong binding interaction was observed between pro-NGF and the immobilized sortilin.

23. Thus, it is my view that the results indicate that the anti-sortilin antibody was effective in inhibiting binding between pro-NGF and sortilin.

24. It is my view that taken together, the *in vivo* cell survival data provided in Appendix A and the *in vitro* binding data provided in Appendix B illustrate the potential beneficial clinical effects of using an antibody to inhibit binding between a pro-neurotrophin and the sortilin receptor for treating an animal having an injury or dysfunction of the central or peripheral nervous system.

25. Furthermore, based on the homology of members of the Vps10p-domain receptor family, I would expect that results similar to that illustrated in Appendix A (*in vivo* data) and in Appendix B (*in vitro* data) are possible for other Vps10p-domain receptors. A person of

ordinary skill and level of knowledge in the field would come to the same conclusions as I have based on the data presented by the Applicants.

26. I understand that specific amino acid sequences directed to a family of receptors containing the Vps10p-domain have been identified.

27. Once provided with a specific sequence (which may encode a specific antigen or a specific epitope of an antigen), polyclonal and monoclonal antibodies directed against the specific sequence can be produced according standard procedures (Harlow and Lane. 1998. Antibodies – A Laboratory Manual. Cold Spring Harbor Laboratory, ISBN 0-87969-314-2).

28. Accordingly, based on the available sequence information, it would be routine to develop a specific antibody against a specific sequence.

29. An antibody can be administered *in vivo* to a site of injury in the central and peripheral nervous system by direct means. This is well known and used by those of skill in the art.

30. For example, intrathecal administration of antibodies is well-known in the art and used to treat cancers such as gliomas and melanomas (Bigner *et al.* 1995. *Journal of Neuro-Oncology* 24(1):109-122), CNS lymphomas (Rubenstein *et al.* 2003. *Blood* 101(2):466-468), and leptomeningeal tumors (Lashford *et al.* 1988. *Cancer* 61:857-868) as well as experimental autoimmune encephalitis (French and Tschopp. 2003. *Cell Death and Differentiation* 10:117-123).

31. In addition, intracranial administration of antibodies has been investigated for reduction of β-amyloid deposition (Alzheimer's Disease) (Wilcock *et al.* 2003. *J Neurosci* 23(9):3745-3751).

32. Furthermore, it is well known in the art that compounds that normally do not cross the blood-brain barrier can be tethered, conjugated or packaged to other components that facilitate the crossing of the barrier. *See*, for example, Friden *et al.* (1991. *PNAS* 88(11):4771-4775).

33. In the event that an antibody is administered by peripheral means, routine techniques can be used to facilitate the delivery of the antibody across the blood brain barrier.

34. For example, an antibody can be genetically engineered and expressed as a fusion protein, in which the antibody is fused with a blood brain barrier "molecular Trojan horse" (Pardridge. 2007. *Pharm Res* 24(9): 1733-1744).

35. In addition, an antibody can be cationized by covalent modification to allow it to penetrate the blood brain barrier (Bickel *et al.* 1995. *Advanced Drug Delivery Reviews* 15:53-72; Triguero *et al.* 1989. *PNAS* 86:4761-4765).

36. It is my view that while the amount of experimentation needed to geneticially or chemically modify the antibody may be high, it would involve standard protocols and routine experimentation that are well known to those of ordinary skill in the art.

37. With respect to the ability of antibodies to cross the blood-brain barrier, it is well known in the art that injury or neurodegeneration in the central and peripheral nervous system can cause the blood-brain barrier to become leaky.

38. During pathological conditions, the permeability of the blood brain barrier increases (Ballabh *et al.* 2004. *Neurobiology of Disease* 16:1-13).

39. For example, disruption of the blood brain barrier can be a major part of the pathology following cerebral ischemia, and leaking of the blood-brain barrier during such injury or neurodegeneration caused by ischemia can allow passage of compounds that are normally too large to cross the barrier, including antibodies (Rubin and Staddon. 1999. *Annu Rev Neurosci* 22:11-28).

40. In addition, many brain diseases change the functionality and integrity of the blood brain barrier. Thes effects typically result in increased blood brain barrier permeability (De Boer and Gaillard. 2006. *J. Neural Transm* 113:455-462).

41. For example, development of Alzheimer's Disease may be related to impairments of the barrier function of the blood brain barrier, indicating that the blood brain barrier may have been breached prior to diagnosis of the disease (Arshavsky. 2006. *J Neural Transm* 113:1697-1707).

42. Further, many diseases having an inflammatory component (*e.g.* multiple sclerosis, meningitis, encephalitis, ischemic stroke, head trauma, neurodegenerative diseases, AIDS-related dementia) change the functionality and/or integrity of the blood brain barrier and, accordingly, brain homeostasis (De Boer and Gaillard. 2006. *J. Neural Transm* 113:455-462).

43. In multiple sclerosis, increased blood brain barrier permeability is an early event. Furthermore, the barrier is typically disrupted as a consequence of injury to the nervous system, such as stroke (Neuwalt. 2004. *Neurosurgery* 54:131-142).

44. Thus, an antibody could cross the blood brain barrier in an animal having an injury or dysfunction in the central or peripheral nervous system even without being engineered to do so.

45. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

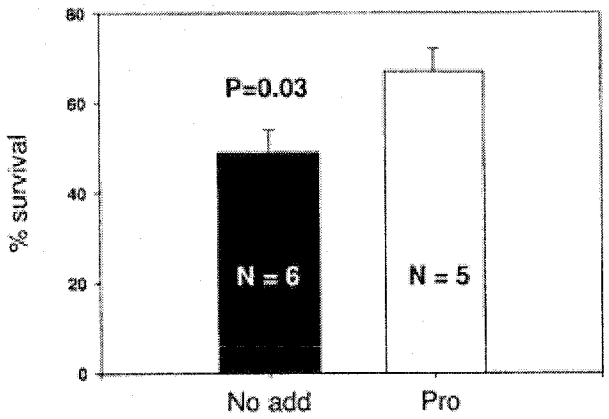
Respectfully submitted,

Dated: Helsinki, June 8, 2009

By: _____
Eero Castrén

## Appendix A

In an experiment, male rats were subjected to spinal cord injury as described by Harrington *et al.* (2004. *Proc.Natl.Acad.Sci* 101 :6226-6230), in the absence (black column in figure below) or the presence of 1 ml/hrs of 100 mg/ml propeptide of proNGF (white column in the figure below). The receptor antagonist was applied by using osmotic minipumps for 7 days, after which the number of surviving motor neurons were scored. The propeptide of proNGF was fused to GST as described in Nykjaer *et al.* (2004. *Nature* 427:843-848).




The results illustrated in the above figure demonstrate that whereas only 49% of the neurons survive in the absence of a sortilin antagonist, 67% of the neurons stay alive in the presence of the proNGF propeptide, which specifically inhibits binding of proneurotrophins to sortilin.

Appendix B

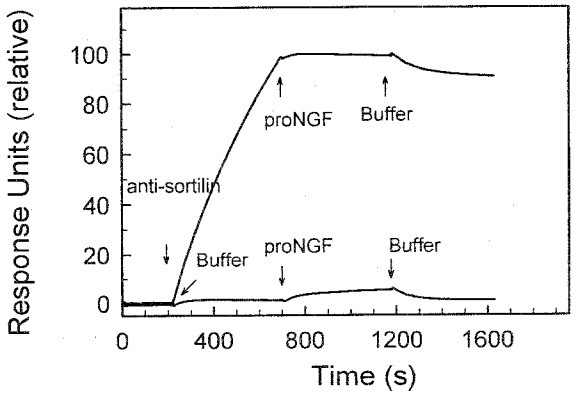
**Material and methods**

Recombinant human sortilin (Quistgaard et al, Nat. Struc. Mol. Biol. (2009) 16: 96-98) was immobilized on a BIAcore CM5 sensorchip at a density of 0.078 pmol/mm.

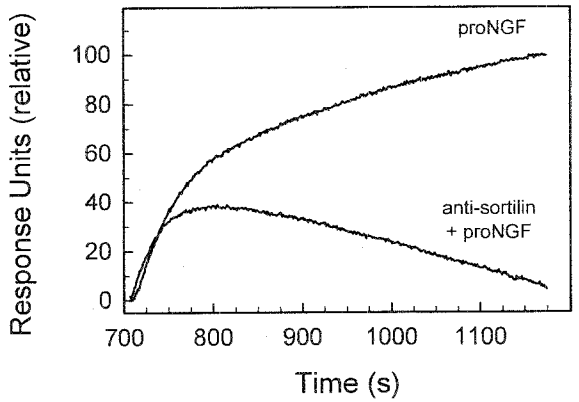
A) After baseline calibration in running buffer (10mM Hepes, 150mM NaCl, 1mM EGTA, 1.5mM CaCl2, 0.005% P20, pH 7.4) 10ug/ml rabbit anti -sortilin IgG (Nykjaer et al, Nature (2004) 427:843-848) or running buffer alone was applied to the chip. At t = 700 sec the response units obtained in the presence of anti-sortilin IgG was arbitrarily set at 100 and 50 nM proNGF was applied to the flow cell pre-incubated with antibody or with buffer alone. Association of proNGF binding was measured until 1200 sec after which the solute was changed to buffer to allow dissociation.

B) Data extracted from panel A. Binding of proNGF at t = 1200 sec to the flow cell following pre-incubation with running buffer alone (=maximum proNGF binding) was set at 100 relative response units. When pre-incubated with the inhibitory anti-sortilin antibody, no proNGF binding was observed.

A


Response Units (relative)
100
80
60
40
20
0
anti-sortilin
Buffer
proNGF
Buffer
proNGF
Buffer
0
400
800
1200
1600
Time (s)

B


Response Units (relative)
proNGF
100
80
60
40
20
0
anti-sortilin
+ proNGF
700
800
900
1000
1100
Time (s)